AD_____

Award Number: W81XWH-11-1-0205

TITLE: A Unique Opportunity to Test Whether Cell Fusion is a Mechanism of Breast Cancer Metastasis

PRINCIPAL INVESTIGATOR: Brenda Ogle

CONTRACTING ORGANIZATION: University of Minnesota-Twin Cities
Minneapolis, MN 55455-2009

REPORT DATE: June 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
June 2015	Annual	7/1/14 – 5/31/15

4. TITLE AND SUBTITLE

A Unique Opportunity to Test Whether Cell Fusion is a Mechanism of Breast Cancer Metastasis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0205

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Brenda Ogle, Caroline Alexander, Ty Harkness, F. Noubissi

email: ogle@umn.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Minnesota-Twin Cities
Minneapolis, MN 55455-2009

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The goal of this proposal is to determine whether cell fusion between tumor cells and hematopoietic cells is the precipitating event for breast cancer metastasis and whether viral fusion proteins enable or catalyze this event. If successful, this discovery would dramatically change our approach to breast cancer therapy in the following specific ways. To date we have completed a significant portion of the tasks delineated in Aim 1**.** First we have optimized protocols for the separation of myeloid and monocyte populations from human mononuclear cell populations. Also we have optimized electroporation conditions for T47D and human mesenchymal stem cell populations. As a result we have been able to conduct our first co-culture experiments to determine whether breast cancer cells fuse spontaneously with hematopoietic cell types. Preliminary results suggest these populations do fuse spontaneously and that fusion products formed in this way can survive several days and are capable of proliferation. In the upcoming months, combinatorial co-cultures with different blood cell subpopulations (myeloid, lymphoid, and monocyte) and healthy or diseased mammary lines will be performed to determine the frequency of fusion between these subpopulations. The effect of hypoxia and hypoglycemia on the frequency of fusion will also be determined. Identified fusion products will then be purified via flow cytometry and assessed for the capacity to migrate and proliferate.

15. SUBJECT TERMS

None Listed

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	17	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

INTRODUCTION

Ninety percent of breast cancer-related deaths are due to secondary tumors, or metastases, that form at sites far removed from the primary tumor. Exactly how metastases form is yet unclear. The goal of this proposal is to determine whether cell fusion between tumor cells and hematopoietic cells is the precipitating event for breast cancer metastasis and whether viral fusion proteins enable or catalyze this event. If successful, this discovery would dramatically change our approach to breast cancer therapy in the following specific ways. First, the studies proposed here will help identify fusion partner(s) able to fuse with breast cancer cells to promote a metastatic phenotype. With this information, it may be possible to design strategies to limit interaction with breast cancer cells, including removal of the cell type. In addition, future studies could identify the specific receptor-ligand interactions necessary for cell fusion, to produce a target for drug therapy. Post-fusion events might also be investigated, including the molecular steps governing the integration or rearrangement of genomic DNA to form a single hybrid genome or those steps necessary for activation of genes that regulate the migratory or invasive phenotype. Second, the studies proposed here will investigate the possibility that exogenous, virus-associated proteins might facilitate breast cancer cell fusion. If viral fusogens are found to promote tumor cell fusion, viral vaccination regimes may be appropriate as a prevention strategy. Vaccines might be developed to target viral fusion genes (i.e., fusogens) exclusively, so that the immune system would recognize the protein, even in the context of a eukaryotic cell membrane. Third, the studies proposed here will establish new tools for the study of the complex processes of cell fusion. The inducible bipartite nature of these strategies assures the accurate identification of fusion products, and allows for longitudinal assays both *in vitro* and *in vivo.*

BODY

The research accomplishments made to date relate to both Specific Aims 1 and 2. Here we reiterate the aims and describe progress on tasks related to the aims as they were delineated in the original statement of work. Of note, the majority of the tasks of Aim 1 are complete. We found that breast cancer cells (and normal breast epithelial cells) can fuse spontaneously and reliably with mesenchymal stem cells. We found that fusion occurs more frequently with hypoxia and that one means by which hypoxia enables fusion is via signaling associated with apoptotic cells. These results were recently published in *FASEB Journal*.

Specific Aim 1. To determine the specificity and functional capacity of hematopoietic cells that spontaneously fuse with breast cancer cells in vitro.

Aim 1a. Tasks 1-7 have been successfully completed.

Aim 1b. Tasks 1-3 have been successfully completed.

Task 4 – Determine invasive capacity of fusion products using long-term imaging and a modified Boyden migration assay chamber (near completion, ~2 mos remaining)

We are developing an inverted vertical invasion assay capable of assessing the invasion capabilities of fusion products in a three dimensional environment. Our results show that mesenchymal stem cells, which are one of the main fusion partners of cancer cells, invade vertically into the assay we are developing when under the influence of growth factors chemoattractants. This assay will aid in analyzing the metastatic capability of both cancerous cells and associated fusion hybrids and help understand better the mechanisms of metastasis

with the hope of developing new therapeutic targets to inhibit cancer cell fusion and metastatic spread.

Approach: **Current design allows cells to remain in original microenvironment.** We attempted to perform assays in several different plate designs, but ultimately concluded that a 35 mm MatTek dish with a 10 mm glass bottom well is the most effective plate for the inverted vertical invasion assay (Fig. 1). Other attempts were made using 24-well plate wells, and glass bottom chamber slides. The advantage of the MatTek dishes comes from several properties. First, the glass bottom of the wells allow for clear and distinct images when compared with those of the standard plastic bottom. Second, the 10 mm well allows for a thin layer of collagen gel to be overlaid atop the cells. Other plate designs required a thicker gel layer that eventually killed the cells beneath due to the lack of oxygen diffusion through the gel. Most importantly, the current design allows the cells to remain within the original microenvironment, unlike the more commonly used Boyden chamber (Fig. 1a).

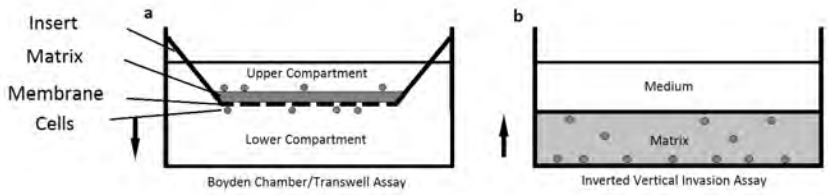

Figure 2 a) Traditional Boyden chamber or transwell invasion assay. This assay requires that the cells are collected from the original culture location and plated onto the transwell insert. The bottom of the insert is a porous membrane which allows for cell migration into the underlying medium. To measure invasion versus migration the porous membrane is coated with a layer of extracellular matrix protein. **b)** Inverted vertical invasion assay currently proposed. This design allows the cells to remain within the original microenvironment. The layer of matrix protein is cast atop the cell layer. After polymerization, medium is overlaid atop the matrix layer. Confocal imaging is used to assess the number of cells invading into the collagen matrix.

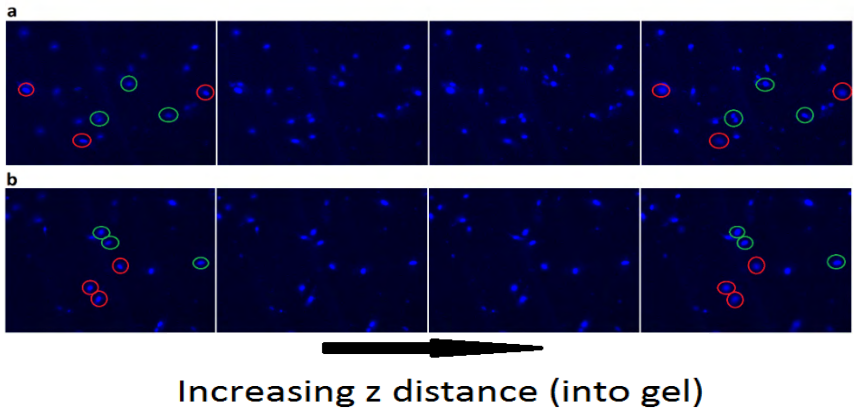

Figure 1 a-b) Z series of two locations within a dish following three days. Each successive image is 24µm above the previous. 3.8% FBS was used as a chemoattractant within the gel. Red and green circles aid in the tracking of individual cells along the z axis. Green circles indicate putative invading cells; note, in focus (right-most image) invading into gel, out of focus (left-most image) beneath gel.

Assessment of chemoattractant to promote invasion. Originally, we attempted the assay using 20% FBS in the α-MEM used to formulate the collagen gels. Thus, after diluting the growth medium into the collagen gel, the gel layer consisted of approximately 3.8% FBS. Images of the assay three days after plating the collagen gel showed that very few MSCs invaded into the gel matrix (Fig. 2). Days one and two presented with even fewer invading cells (not shown). The use of IGF-1 and PDGF-BB as chemoattractants within the gel showed increased invasion on the part of MSCs. We performed the assay using both insulin-like growth factor-I (IGF-I) and platelet-derived growth factor-BB (PDGF-BB). We compared the percentage of cells invading to that when performing the assay using only FBS (Fig. 3). The concentrations used were based off a previous study that determined the optimum growth factor concentrations for optimum MSC chemotaxis. Results showed that the 18.4 ng/mL concentration of IGF-I

produced significantly improved invasion when compared to the assay using solely 20% FBS. Platelet-derived growth factor produced invasion similar to that of solely 20% FBS.

Table 1. Invasive capacity of MSCs

Experiment	Average Percent Invading Cells	Std. Dev.
FBS 3.8%	5.63	2.35
IGF-I (18.4 ng/mL)	43.5	28.3
IGF-I (13.8 ng/mL)	8.78	4.07
IGF-I (9.2 ng/mL)	8.51	18.9
PDGF-BB (3.7 ng/mL)	5.95	5.11
PDGF-BB (2.8 ng/mL)	1.21	0.94

Our results here show that the proposed design of the inverted vertical invasion assay should be a viable tool in the measurement of invasion of cancer cell fusion product invasion. The design has proven that viable cell cultures can be maintained for up to a minimum of three days under the collagen gel matrix. Likewise, the gel integrity is maintained for just as long, except in the case of excess motion or other disturbances to the culture. We have also shown that the inverted invasion assay in the glass bottom MatTek dishes allow for clear z-stack images to be taken of the collagen gel, and thus cell invasion can be noted. We did show that the assay requires a stronger a chemoattractant than fetal bovine serum, most likely due to the preferable environment on the glass-bottom of the well. When we performed the assay using insulin-like growth factor-1, a known strong chemoattractant of MSCs, we saw that a significantly higher percentage of the MSCs invaded into the gel. While not shown above, it was seen that in the assays using growth factors that many MSCs would invade in excess of 300 micrometers into the collagen gel. Thus, the growth factors not only increased the number of invading cells, but also the distance of which they invaded.

(Last Reporting Period Problem Resolved) Unanticipated challenges of the BiFC plasmids

Last reporting period, we noted the BiFC probes were found to inhibit proliferation and long term viability of certain fusion partners. To avert this problem we successfully deleted Histone H3.1 coupling from one half of the BiFC probe set. With this approach proliferation and cell viability long term were improved.

Specific Aim 2. To determine whether tumor cell fusion products give rise to metastatic tumors.

Aim 2a - Tasks 1-3 have been successfully completed.

Aim 2b. Tasks 1-2 have been successfully completed.

We are currently optimizing lentiviral transduction of breast cancer cells isolated from autochtonous mice (Tasks 3, 6). We routinely accomplish >85% DNA transfer efficiency. We also await ACURO approval (as a function of grant transfer) such that Tasks 4, 5 and 7 can be completed.

KEY RESEARCH ACCOMPLISHMENTS

- Optimization of MACS magnetic bead separation of myeloid and monocyte populations from human buffy coat (previous report)
- Optimization of electroporation transduction conditions for T47D cells (previous report)
- Optimization of lentiviral constructs for BiFC transfection (previous report)
- Engineering of new BiFC pairs to promote viability and proliferative capacity of all cell types of interest for this proposal (previous report)

- Indication of spontaneous fusion between MCF10A, MDA-MD-231 and hMSCs and between T47D and hMSCs in *in vitro* cocultures (previous report)
- Fate of putative T47DxhMSC fusion products tracked over time; some fusion products were found to undergo proliferation (previous report)
- The course of fusion products after transplantation of MSCs expressing the floxed luciferase to cre mice was tracked. (previous report)
- Developed a 3D invasion assay to determine metastatic potential *in vitro.* (this report)
-

REPORTABLE OUTCOMES

Manuscript:
Harkness, TE; Weaver, BA; Alexander, CM; Ogle, BM. Cell Fusion in Tumor Development: Accelerated Genetic Evolution. *Critical Reviews in Oncogenesis*. 18(1-2):19-42. 2013.

Noubissi, FK; Harkness T; Alexander CM; Ogle BM. Apoptosis-induced cancer cell fusion: a mechanism of breast cancer metastasis. *FASEB Journal.* 2015 Jun 17. pii: fj.15-271098. [Epub ahead of print]

Degrees awarded:
Ty Harkness, M.S., University of Wisconsin-Madison.

Employment received: Ty Harkness, Research Scientist, Pfizer, San Francisco, CA; Felicite Noubissi, Assistant Professor, University of Mississippi-Jackson.

Research tools developed: Bimolecular fluorescence complementation lentiviral vectors for study of cell fusion *in vitro*; specifically, VN-H3.1, YC-H3.1 and VN-null. Floxed luciferase lentiviral vectors for study of cell fusion *in vivo*. 3D cell invasion assay, with special applicability to sensitive cultures such as those associated with cell fusion.

CONCLUSIONS AND FUTURE STUDIES

In the upcoming months we expect to complete and publish two studies. One related to the invasive quality of breast cancer cell hybrids *in vitro* and associated mechanism of fusion related to apoptosis and signaling associated with phosphatidyl serine receptors. The second manuscript will share results associated with *in vivo* studies designed to test whether breast cancer cells can fuse at the primary tumor site and whether fusion of this type can enable metastasis. If the hypothesis holds, we expect this to garner significant attention in the field.

SUPPORTING DATA

Find attached reprint for manuscript published in this reporting period.

The FASEB Journal article fj.15-271098. Published online June 18, 2015.

Apoptosis-induced cancer cell fusion: a mechanism of breast cancer metastasis

Felicite K. Noubissi,*,† Ty Harkness,‡ Caroline M. Alexander,‡,§ and Brenda M. Ogle*,†,‡,¶,‖,1

*Department of Biomedical Engineering, ¶Stem Cell Institute, ‖Lillehei Heart Institute, †Masonic Cancer Center, University of Minnesota-Twin Cities, Minneapolis, Minnesota, USA; and ‡Department of Biomedical Engineering and §Department of Oncology, University of Wisconsin-Madison, Madison, Wisconsin, USA

ABSTRACT **Although cancer cell fusion has been suggested as a mechanism of cancer metastasis, the underlying mechanisms defining this process are poorly understood. In a recent study, apoptotic cells were newly identified as a type of cue that induces signaling *via* phosphatidylserine receptors to promote fusion of myoblasts. The microenvironment of breast tumors is often hypoxic, and because apoptosis is greatly increased in hypoxic conditions, we decided to investigate whether the mechanism of breast cancer cell fusion with mesenchymal stem/multipotent stromal cells (MSCs) involves apoptosis. We used a powerful tool for identification and tracking of hybrids based on bimolecular fluorescence complementation (BiFC) and found that breast cancer cells fused spontaneously with MSCs. This fusion was significantly enhanced with hypoxia and signaling associated with apoptotic cells, especially between nonmetastatic breast cancer cells and MSCs. In addition, the hybrids showed a significantly higher migratory capacity than did the parent cells. Taken together, these findings describe a mechanism by which hypoxia-induced apoptosis stimulates fusion between MSCs and breast tumor cells resulting in hybrids with an enhanced migratory capacity that may enable their dissemination to distant sites or metastases. In the long run, this study may provide new strategies for developing novel drugs for preventing cancer metastasis.—Noubissi, F. K., Harkness, T., Alexander, C. M., Ogle, B. M. Apoptosis-induced cancer cell fusion: a mechanism of breast cancer metastasis. *FASEB J.* 29, 000–000 (2015). www.fasebj.org**

Key Words: hybrid · hypoxia · migration · mesenchymal stem cell · bimolecular fluorescence complementation

FUSION OF BREAST CANCER CELLS with cells of the tumor microenvironment has been proposed as a means of generating widespread genetic and epigenetic diversity (1–4). Diversity created in this way could rapidly enhance the formation, propagation, and metastasis of tumor cells or could quickly alter drug sensitivity. Yet to be reconciled are the precise mechanisms governing hybrid reprogramming after cell fusion and the means by which cancer cells undergo spontaneous fusion in the first place. The latter was the focus of this work.

One way that spontaneous cell fusion is enabled is *via* specific integral membrane proteins, termed fusogens, which provide cell anchorage and disrupt cell membranes, to minimize the energy necessary to overcome the merger of the 2 hydrophobic membranes (5). The structure and function of many viral fusogens are well characterized, but eukaryotic fusogens are more difficult to establish because removal of facilitating proteins from the system reduces overall fusion rates. As technologies advance, many putative fusogens have been contested and shown to be merely adhesion proteins that bring cell membranes in close apposition but do not actually facilitate fusion. Take, for example, heterotypic gamete fusion. As a fusion product, the fertilized embryo can proliferate and differentiate into all the tissues of the adult body as well as the extraembryonic tissues. CD9 is an egg-associated putative fusogen. Cluster of differentiation (CD)9-knockout mice have shown severely hampered fertilization (6) that is restored with polyadenylated CD9 mRNA (7). In addition, CD9 has been shown to generate the strongest observed interactions with the sperm (8). However, it is not known whether it specifically facilitates the disruption and merging of membranes or whether other facilitating proteins or conditions are necessary (5).

Bona fide fusogens may not be necessary for cell fusion if cell membranes can be disrupted by other means. Cell stress, including nutrient deprivation, electroporation, and hypoxia, can render cell membranes leaky or unstable (9–11). For example, changes in the ratio of cholesterol to phospholipid and in the double-bond index of the associated fatty acids have been shown to alter plasma membrane structure during hypoxic injury (12). In addition, alterations in lipid structure with hypoxia can lead to changes in membrane protein structure and function, resulting in dysregulated enzyme activity and transport properties that could also lead to leakiness of the plasma membrane barrier (13). Unstable cell membranes are biophysically

Abbreviations: αMEM, α-minimum essential medium; BAI, brain-specific angiogenesis inhibitor; BiFC, bimolecular fluorescence complementation; CCL, chemokine ligand; CD, cluster of differentiation; CMV, cytomegalovirus; COI, cytochrome *c* oxidase I; Dock, dedicator of cytokines; EBV, Epstein-Barr virus; ELMO, engulfment cell and mobility protein; GFP,

(*continued on next page*)

[1] Correspondence: University of Minnesota-Twin Cities, 312 Church St. SE, Minneapolis, MN 55455, USA. E-mail: ogle@umn.edu

doi: 10.1096/fj.15-271098

This article includes supplemental data. Please visit *http://www.fasebj.org* to obtain this information.

susceptible to membrane fusion (14, 15). It may be for this reason that cell fusion appears to occur more readily in the context of hypoxia than during normoxia (16, 17). In contrast, cell fusion may be enhanced with hypoxia *via* signaling events triggered by hypoxic stress. For example, human hematopoietic progenitor cells upregulate VCAM-1 with hypoxia, a protein found to enhance adhesion and fusion with cardiomyocytes. Similarly, fusion of skeletal myocytes is increased during hypoxia, in part because of signaling events associated with apoptotic cells. Apoptotic cells exhibit exposed phosphatidyl serine and choline, which binds associated phosphatidyl serine and choline receptors expressed by skeletal muscle. Binding of these receptors in skeletal muscle can engage the engulfment cell and mobility protein (ELMO)/dedicator of cytokines (Dock)180/ras-related C3 botulinum toxin substrate (Rac)-1 module to enhance myoblast fusion (18).

To accurately test whether apoptotic cells enable cell fusion, we used a unique tool developed in our lab to detect fusion products that was based on bimolecular fluorescence complementation (BiFC). With this approach, a fluorescent signal is induced by hybrid formation and can be detected in living cells. In addition, the signal intensity in hybrids increases over time as BiFC products are continuously synthesized. Using this robust tool, we showed that spontaneous fusion of multipotent stem/stromal cells (MSCs) with breast cancer cells occurs more readily in hypoxia than in normoxia, a significant finding, given that the microenvironment of breast tumors is often hypoxic. In addition, we found that, like skeletal muscle, cell fusion with hypoxia is enhanced as a consequence of the signaling associated with apoptotic cells. These findings are among the first to address how cancer cells spontaneously fuse in the first place and, as a consequence, reveal novel untapped therapeutic targets.

MATERIALS AND METHODS

Cell lines and culture

MSCs were a generous gift from Dr. Peiman Hematti (University of Wisconsin, Madison, WI, USA). They were derived from human embryonic stem cells in accordance with guidelines of the University of Wisconsin Institutional Review Board (19) and maintained in α-minimum essential medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA). We reconfirmed the identity of the MSCs in our lab by flow cytometry for specific MSC markers. Human mammary epithelial cells (MCF10a), human ductal breast epithelial tumor cells (T47D), human breast adenocarcinoma cells (MDA-MB-231), and human breast cancer cells (MCF7) (MCF indicates the Michigan Cancer Foundation where the MCF10a and MCF7 lines were established) were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in accordance with the recommendations of ATCC; the cells were not passaged for more than 6 mo from the time of receipt. MCF10as were tested for: 1) mycoplasma by DNA stain and agar culture, 2) species determination by short tandem repeat (STR) analysis and cytochrome *c* oxidase I (COI) assay, 3) sterility by BacT/ALERT 3D (bioMérieux, St. Louis, MO, USA), and 4) the human pathogens HIV, hepatitis B and C (HepB, HepC), human papilloma virus (HPV), Epstein Barr virus (EBV), and cytomegalovirus (CMV). T47Ds were tested for: 1) mycoplasma by DNA stain and agar culture, 2) species determination by STR and COI assays, and 3) sterility by BacT/ALERT 3D. MDA-MB-231s were tested for: 1) mycoplasma by DNA stain and agar culture, 2) species determination by STR and COI assay, 3) sterility by BacT/ALERT 3D, and 4) the human pathogens listed above. MCF7s were tested for: 1) mycoplasma by DNA stain and agar culture, 2) species determination by STR, 3) sterility by BacT/ALERT 3D, and 4) the human pathogens listed above.

BiFC approach

BiFC is a method of viewing living hybrid cells (20, 21) (Supplemental Fig. S1*A*). Thus, the fluorescence of green fluorescent protein (GFP) is reconstituted when 2 nonfluorescent halves, expressed in fusion partners, are coexpressed and are in proximity in fusion products (22–26). To encourage proximity, each nonfluorescent half is coupled to histone 3.1 (H3.1; plasmids, a generous gift of Dr. Thomas Kerppola, University of Michigan, Ann Arbor, MI, USA). Fluorescence was detected and recorded *via* traditional fluorescence microscopy (excitation 485, emission 530; Olympus IX81; Olympus America, Center Valley, PA, USA). Because of the conditional nature of the signal (*i.e.,* fluorescent signal is detected only after a fusion event), the incidence of false positives was significantly reduced. A fluorescence signal was deemed positive if it was localized to the nucleus (as a consequence of the H3.1 fusion protein), fluoresced above the background fluorescence of unaltered cocultures, and did not fluoresce with excitation/emission at alternate wavelengths. Under these criteria, transduction of the cells with each BiFC half alone did not yield a detectable false-positive signal (Supplemental Fig. S1*B*).

Coculture protocol

The design of coculture experiments is illustrated in Supplemental Fig. S2*A*. In our study, we transduced MSCs with lentiviral particles expressing the BiFC complex amino terminus GFP, histone H3.1 (VNH3.1), and the breast epithelial cells (MCF10a) or breast cancer cells (T47D, MDA-MB-231, and MCF7), with the corresponding BiFC complex carboxyl terminus GFP, histone H3.1 (YCH3.1) (Supplemental Fig. S2*A*). The histone H3.1 tagged to each construct brought the BiFC halves in close association in the nucleus. MSCs were mixed with each epithelial cell type (*i.e.,* MSC with MDA-MB-231, MSC with T47D, MSC with MCF7, and MSC with MCF10a) in 4-well chamber slides, such that the stromal population was mixed with each epithelial cell type. The ratio of cells for cocultures was 80,000 MSCs to 35,000, 112,000, 100,000, and 90,000 MDA-MB-231, T47D, MCF7, and MCF10a cells, respectively. Cell densities were selected so that surface area coverage for each cell type was approximately 50% at 24 h after the cocultures were initiated (Supplemental Fig. S2B). Forty-eight hours after coculturing began, the cells were fixed and stained with DAPI. Fusion products were identified by fluorescence microscopy. A signal (green fluorescence) was considered positive if it was stronger than the background in the FITC channel for unaltered cocultures, was nuclear (overlapped with DAPI), and did not

(*continued from previous page*)

green fluorescent protein; HepB, hepatitis B; HepC, hepatitis C; HPV, human papillomavirus; HSD, honest significant difference; MCF7, human breast cancer cells; MCF10a, mammary epithelial cells; MDA-MB-231, human breast adenocarcinoma cells; MSC, multipotent stromal/mesenchymal stem cell; NOD, nonobese diabetic; PtSer, phosphatidylserine; Rac, ras-related C3 botulinum toxin substrate; STR, short tandem repeat; T47D, human ductal breast epithelial tumor cell; VNH3.1, amino terminus GFP, histone H3.1; YCH3.1, carboxyl terminus GFP, histone H3.1; Z-VAD-FMK, carbobenzoxy-valyl-alanyl-aspartyl-[*O*-methyl]-fluoromethylketone

crossover to the rhodamine and cyanine (Cy)5 channels. The frequency of fusion products was reported relative to the total number of nuclei in at least 200 fields of view per experiment, with about 110 cells per field. We performed each experiment at least 3 times.

Hypoxic treatment

Hypoxic treatment was performed 16 h after the initiation of cocultures. The cocultures were removed from standard tissue culture conditions (21% O_2, 5% CO_2, and 74% N_2) and put in conditions of oxygen deprivation (2% O_2, 5% CO_2, and 93% N_2) for 3 h. At the end of coculturing (48 h after initiation) the cells were fixed and stained with DAPI, and the number of fusion products was recorded. We recognize that standard tissue culture conditions are actually hyperoxic relative to normal tissue, but for the purposes of this study, we considered the condition of 2% O_2 to be hypoxic.

Apoptotic cell treatment

Apoptosis was induced by treatment of T47Ds with 1 μM staurosporine (Sigma-Aldrich) for 12 h. Annexin V staining determined that 50–75% of the cells were apoptotic. Floating and attached cells were collected and used in the experiments. To investigate the involvement of apoptotic cells in cancer cell fusion, MSCs and T47Ds were transduced, and cocultures were initiated as described above. Sixteen hours after initiation of the cocultures, apoptotic cells were added at a ratio of 1 apoptotic cell for 2 coculture cells. The cocultures were analyzed under normoxic and hypoxic conditions, as described above.

Annexin V apoptosis assay

Breast epithelial cells exposed to low oxygen for 3 h were stained with the ApoAlertAnnexin V-FITC Apoptosis Kit (Clontech, Mountain View, CA, USA), in accordance with the recommendations of the manufacturer. Apoptotic cells were detected with a fluorescence microscope.

Caspase inhibitor treatment

Caspase activity was inhibited in cocultures of fusion partners by using the small molecule, Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[*O*-methyl]-fluoromethylketone; Promega, Madison, WI, USA) and the ability of cells to fuse under normoxic and hypoxic conditions was analyzed. Z-VAD-FMK is a cell-permeable pan-caspase inhibitor that irreversibly binds to the catalytic site of caspase proteases and is widely used to inhibit apoptosis. In our study, each cell population (MSCs, T47D, and MCF7) was transduced with the lentiviral particles expressing the BiFC complex VNH3.1 for MSCs, and the corresponding BiFC complex YCH3.1 for T47Ds or MCF7s, as described above. Twelve hours after transduction, each cell population was treated with Z-VAD-FMK (1 μM) for 1 h, and the cocultures were initiated. The coculture medium was supplemented with Z-VAD-FMK during the entire experiment (36 h), and the cocultures were assessed under normoxic and hypoxic (2% O_2, 5%CO_2, and 93% N_2) conditions, with and without the addition of apoptotic cells. The cells were fixed and stained with DAPI, and the fusion products were recorded.

Time-lapse microscopy

Cocultures for time-lapse microscopy were initiated as described in the protocol above. In brief, MSCs were transduced with lentiviral particles from the BiFC component VNH3.1, and T47D or MCF7s were transduced with lentiviral particles from the BiFC component YCH3.1. At 48 h after initiation of the cocultures, the fusion products were identified by fluorescence microscopy (TE200; Nikon, Inc., Melville, NY, USA). The cells were maintained at 5% CO_2, 37°C, in a humidified atmosphere. Phase-contrast and fluorescent images of hybrids were taken every 5 min for 3 h with a ×20 objective (0.5 NA, plan fluor ×20 phase 1). Images of double-transduced parental cells and of control cells were taken. The paths of migrating cells were analyzed by using the manual tracking and chemotaxis and migration tool in NIH Fiji software (ImageJ; National Institutes of Health, Bethesda, MD, USA) (27). In brief, the center of a given nucleus was selected for each frame. Based on the resultant path created, the software was used to calculate velocity, total distance, and directionality.

For long-term tracking (72 h), MSCs and T47Ds were transiently transfected with mCherry- and GFP-expressing plasmids, respectively. Sixteen hours after transfection, the cocultures were initiated, and fusion products (expressing both red and green fluorescence) were identified 24 h later with a fluorescence microscope. Phase-contrast and green fluorescent images of hybrids were taken every 10 min for 72 h with a ×10 objective in the Citation 3 imaging reader (Bio-Tek, Winooski, VT, USA). The cells were maintained at 5% CO_2 and 37°C, and the medium was replaced every 24 h. At 72 h, a static fluorescence image (green and red) of the tracked hybrid was obtained to confirm the hybrid's identity.

Statistical analysis

All data are presented as means ± SD. When 2 groups were compared, a 2-tailed Student's *t* test was used. When more than 2 groups were compared, ANOVA with Tukey's honest significant difference (HSD) *post hoc* test was used. $P < 0.05$ denoted statistically significant differences.

RESULTS

Breast tumor cells fuse with MSCs spontaneously

Fusion between tumor cells and host cells has been shown by several studies, many of which implicate bone marrow–derived cells of myeloid lineage as host fusion partners [reviewed in (17, 28)]. We propose that fusion of tumor cells with cells of the stroma gives rise to hybrid cells capable of dissemination and new tumor growth. In the initial step of investigating the role of cell fusion in breast cancer metastasis, we examined the ability of MSCs to fuse spontaneously with breast cancer cells with different degrees of aggressiveness (T47D cells, which are nonmetastatic, and MDA-MB-231 cells, which are metastatic) and nontumorigenic MCF10a breast epithelial cells . We have developed a powerful tool for the detection of fusion products *in vitro* that uses BiFC (see Materials and Methods and Supplemental Fig. S1*A*). Because of the conditional nature of the signal, the incidence of false positives is significantly reduced. Moreover, transduction of the cells with each BiFC half did not yield any detectable false positives (Supplemental Fig. S1*B*). Lentiviral particles expressing each half of the BiFC complex were delivered to the cells, and cocultures were initiated as described in Materials and Methods (Supplemental Fig. S2*A*). Fusion products were identified with an epifluorescence microscope. The MSCs fused spontaneously with all breast epithelial cell types (**Fig. 1*A*** and Supplemental Fig. S3*A*) at a fusion rate of ~1:1000 for cancer cell types and ~1:2000 for the

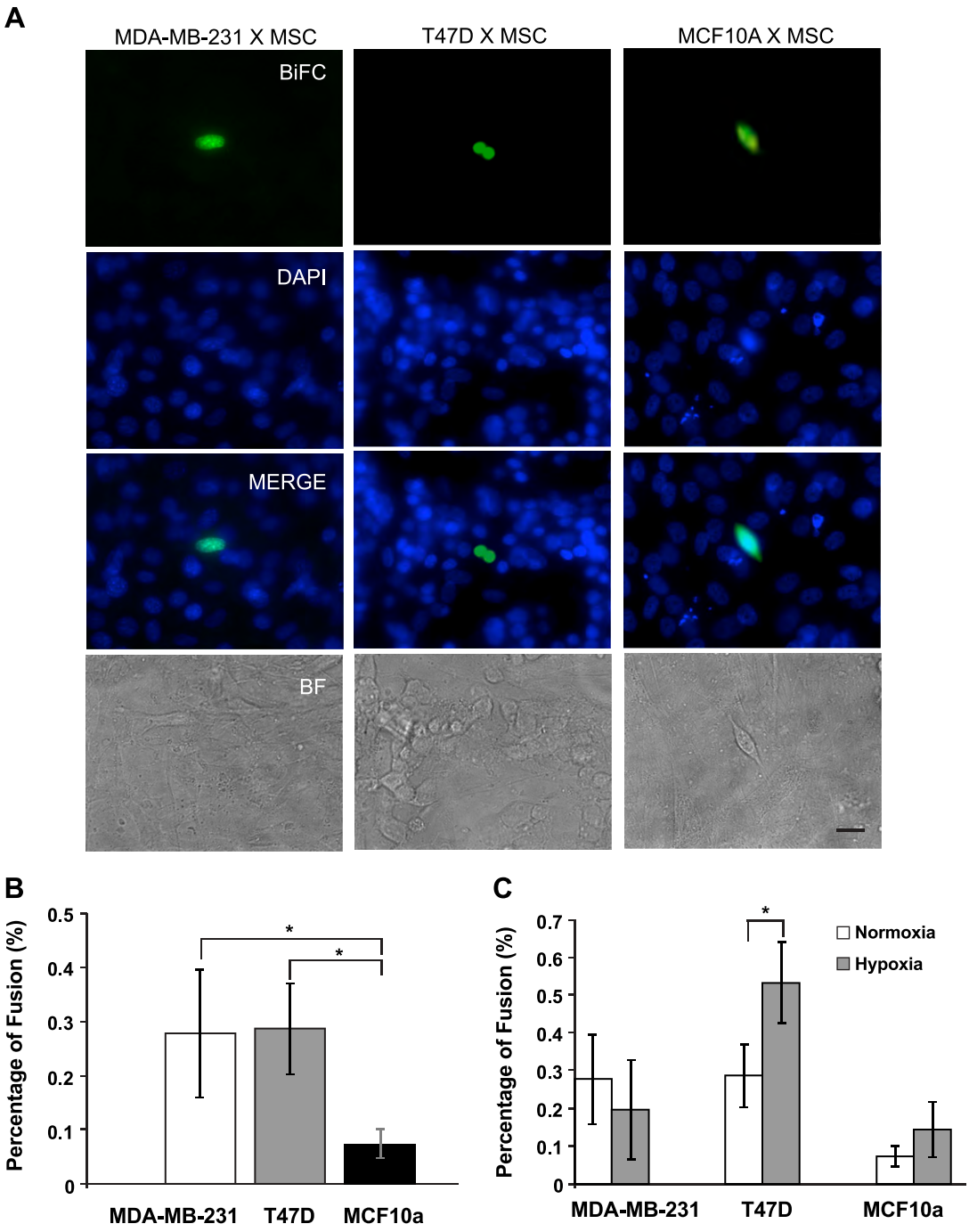

Figure 1. MSCs fuse spontaneously with breast epithelial cells. *A*) Representative fusion products from MSC-breast epithelial cell cocultures. Scale bar, 25 μm. *B*) Frequency of fusion of MSCs with breast epithelial cells. *C*) Percentage of cell fusion between MSCs and MDA-BM-231s, T47Ds, or MCF10as under hypoxic and normoxic conditions. *B*, *C*) Each value (mean ± SD) represents the average of 3 independent experiments performed in triplicate. ANOVA with Tukey's HSD *post hoc* test. $^*P < 0.05$.

nontumorigenic cell line, MCF10a ($P < 0.05$) (Fig. 1*B* and Supplemental Fig. S3*B*).

Fusion is enhanced with hypoxia

To test whether the rate of fusion could be regulated by factors associated with tumor microenvironments, we evaluated the effect of hypoxia (29). This hypoxic microenvironment puts cancer cells under stress and may promote their fusion with cells of the stroma recruited to the tumor microenvironment, as a means of survival. Cells were transduced with lentiviral particles expressing each half of the BiFC complex, and cocultures were initiated between MSCs and breast epithelial cells. The cocultures were subsequently exposed to low oxygen for 3 h, and fusion products were recorded, as described in Materials and Methods. We found that hypoxia stimulates a significant increase in fusion between MSCs and the T47D nonmetastatic breast cancer cells ($P < 0.05$; Fig. 1*C*). This

increase in fusion was not restricted to T47Ds, but was also observed with the nonmetastatic MCF7 cancer cells ($P < 0.05$; Supplemental Fig. S3*B*).

Fused breast tumor cells show increased migratory capability

To study the contribution of spontaneous cell fusion to metastasis, we used time-lapse microscopy to analyze the migratory capability of hybrids derived from the fusion between T47Ds and MSCs. Cocultures were initiated between the MSCs and the T47Ds. Forty-eight hours after the initiation of the cocultures, the hybrids were identified and tracked every 5 min for 3 h with an inverted fluorescence microscope. The MSCs were highly motile and showed directed travel patterns. The T47Ds were 3 times less motile and showed little directional travel. The hybrids acquired the enhanced motility of the MSC parent, but were nondirectional, similar to their T47D breast cancer cell parent (**Fig. 2*A, B***). The same trend in motility and directionality was observed in hybrids between MCF7s and MSCs (Fig. 2*C, D*).

The mechanism of fusion of breast cancer cells involves apoptosis

Given its potential relevance in breast cancer metastasis, it is critical to understand the mechanisms governing cell fusion. In the experiments described above, we noted that hypoxia had little impact on the ability of MDA-MB-231 metastatic cancer cells to fuse spontaneously with MSCs. This observation suggests that hypoxia promotes fusion of nonmetastatic cancer cells with MSCs and therefore enables metastasis, an event that is not necessary or advantageous for the already metastatic MDA-MB-231 cells. A recent study identified signaling *via* the phosphatidylserine (PtdSer) receptor brain-specific angiogenesis inhibitor (BAI)-1 as a cue to promote fusion of healthy myoblasts, involving the ELMO/Dock180/Rac1 pathway (18). Blocking apoptosis potently impaired myoblast fusion, and adding back apoptotic myoblasts restored fusion. The added apoptotic myoblasts exposed PtdSer, but did not attach to the tissue culture plate or fuse on their own. They were necessary to promote myoblast fusion, requiring PtdSer-dependent cell-cell contact between apoptotic and viable myoblasts (18). Because apoptosis is increased in certain cell types in hypoxic conditions [reviewed in (30)], we tested whether the same would be true of the breast epithelial cells used in this study. In fact, apoptosis was enhanced by hypoxia (2% O_2, 3 h) only in the T47D and MCF7 nonmetastatic cancer cells (**Fig. 3**). Because we found that hypoxia considerably increases fusion between MSCs and the T47D and MCF7 breast tumor cells but not between MSCs and MDA-MB-231s or MCF10as (Fig. 1*C* and Supplemental Fig. S3*B*), we investigated first whether apoptosis is involved in the fusion of cancer cells. Cocultures between MSCs and T47Ds were initiated as described in Materials and Methods. Sixteen hours after initiation of cocultures, they were supplemented with T47D apoptotic cells, and fusion products were recorded. We found that addition of apoptotic cells to cocultures significantly increased cell fusion between MSCs and T47Ds in normoxic and hypoxic conditions ($P < 0.05$; **Fig. 4*A***). To continue the investigation of the role of apoptosis in cancer cell fusion, we assessed whether inhibition of apoptosis impairs cell fusion. Apoptosis was inhibited in the fusion partners before coculture initiation with the pan-caspase inhibitor Z-VAD-FMK (1 μM). The inhibitor remained in the coculture medium during the entire coculture experiments and did not seem to affect the morphology or viability of the cells. We analyzed the ability of MSCs and T47D to fuse in normoxic and hypoxic conditions. We found that inhibition of apoptosis reduces cell fusion in normoxic conditions, and this reduction was significant in hypoxic conditions ($P < 0.05$), whereas addition of apoptotic cells rescued the fusion diminished by the pan-caspase inhibitor (Fig. 4*A*). Apoptosis appeared also to regulate the fusion of nonmetastatic MCF7s and MSCs (Fig. 4*B*), suggesting that the mechanism by which cancer cells fuse with MSCs involves apoptosis.

DISCUSSION

Our findings describe a novel mechanism by which cancer cells fuse with MSCs, an event that could underlie the development of metastases. The breast cancer cells fused spontaneously with the MSCs. This fusion was significantly enhanced by hypoxia, especially between the nonmetastatic breast cancer cells and MSCs, and was considerably increased by apoptosis. In addition, the hybrids formed showed a significantly higher migratory capability, lending support to the prediction that fusion contributes to cancer metastasis.

The possibility that cell fusion gives rise to the metastatic phenotype was first put forward nearly a century ago by Aichel (1) and later by Mekler (31) and Goldenberg (32). In recent years, this hypothesis has gained further support. Spontaneous fusion has been reported between normal breast epithelium and breast cancer cells (33–36), among breast tumor cells themselves (37–39), between breast cancer epithelium and endothelial cells (40), and between breast cancer epithelium and tumor stromal cells (12, 13). *In vitro* studies of hybrids formed between normal breast epithelium (M13SV1-EGFP-Neo) and breast cancer cells (HS578T-Hyg) showed increased locomotory activity, compared with the normal parental lines. This fusion-enhanced migration was associated with altered chemokine ligand (CCL)21/chemokine receptor (CCR)-7 signaling, which has been linked to the metastatic spread of breast cancer to lymph nodes (34). Increased metastatic potential of hybrids was also observed *in vivo* when breast cancer cell variants (MDA-MB-231) with tropism for either lung or bone injected in nude mice gave rise to hybrids capable of metastases to both organs (37). Drug susceptibility was also found to be altered in hybrids. Hybrids formed between parental breast cancer cells (MCF-7), with and without resistance to doxorubicin, were heterogeneous, with some exhibiting resistance and others not (39). Likewise, hybrids derived from breast epithelial cells (M13SV1-EGFP-Neo) and breast cancer cells (MDA-MB-435-Hyg) showed altered sensitivity to the PI3K inhibitor, Ly294002, as a consequence of differential RAF-AKT crosstalk among hybrids (36). Close probing of the heterogeneity generated by the formation of stromal

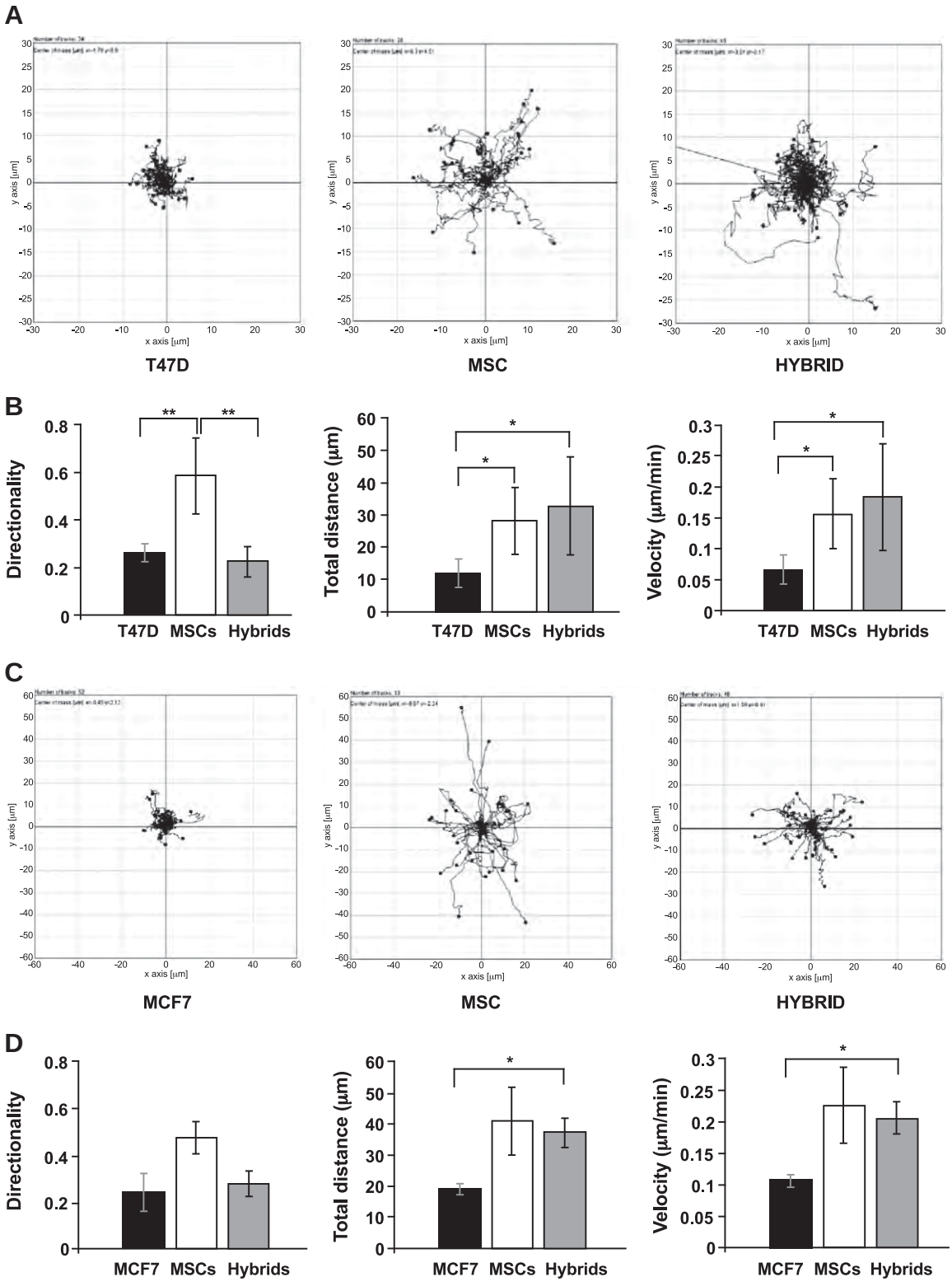

Figure 2. Hybrid cells from MSC-T47D or MSC-MCF7 cocultures show higher migratory capability. Migration of hybrid cells compared with that of MSCs alone or T47Ds alone (time-lapse microscopy). *A*) Representation of motility pattern of T47Ds, MSCs, and hybrids. *B*) Directionality of motion of cells. Total accumulated distance, and velocity. Each value (mean ± SD) represents the average of 4 independent experiments with the analysis of at least 40 cells per group and per experiment. ANOVA with Tukey's HSD *post hoc* test. $*P < 0.05$, $**P < 0.01$. *C*) Representation of the motility pattern of MCF7s, MSCs, and hybrids. Cells of cocultures of MCF7s and MSCs were identified and tracked by time-lapse microscopy. *D*) Hybrids between MCF7s and MSCs showed increased migratory capability. Migration of hybrid cells compared to that of MSCs alone or MCF7s alone (time-lapse microscopy) including directionality of motion of cells, total accumulated distance, and velocity. Each value (mean ± SD) represents the average of 3 independent experiments with analysis of at least 40 cells per group and per experiment. ANOVA with Tukey's HSD *post hoc* test. $*P < 0.05$.

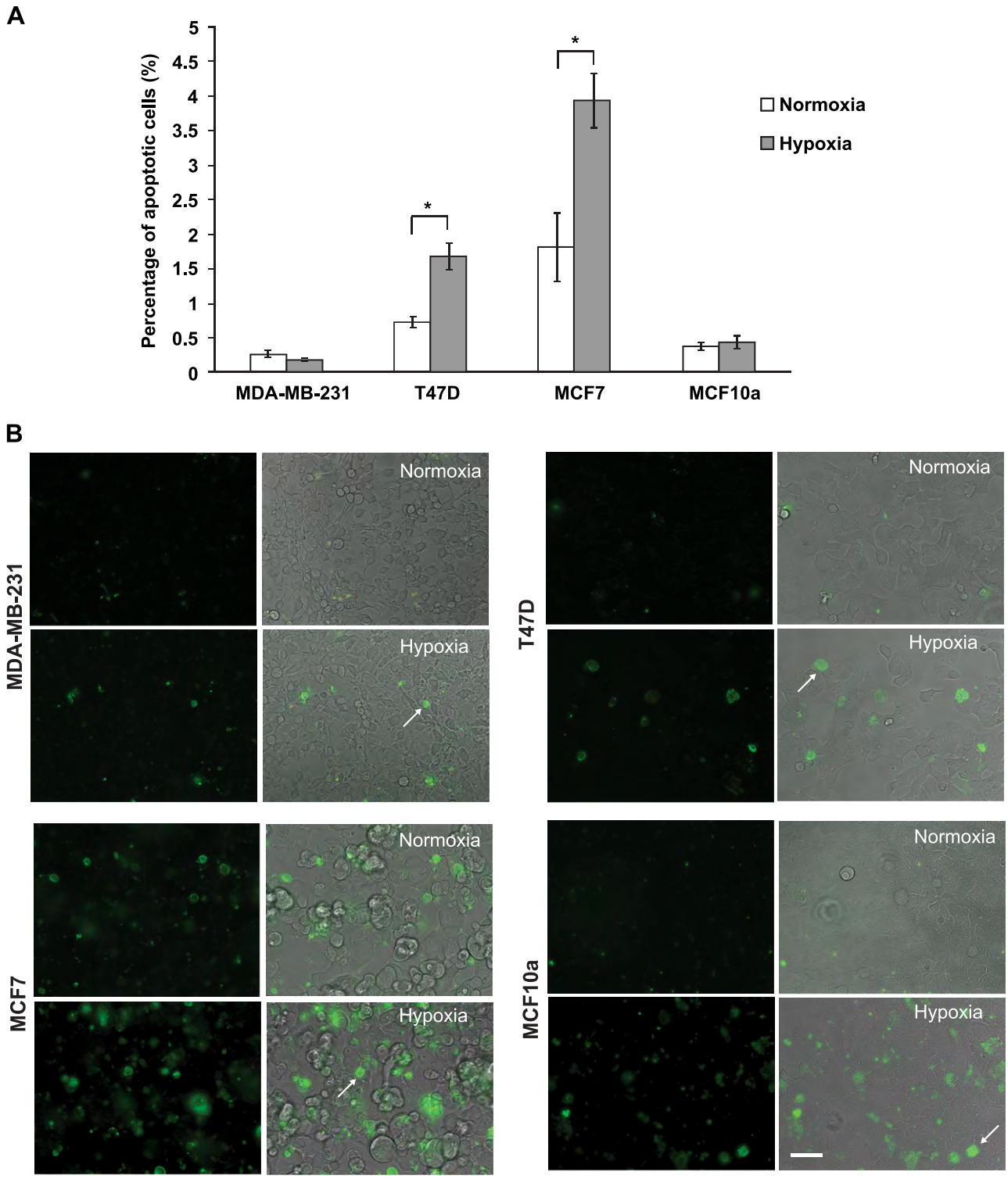

Figure 3. Hypoxia-induced apoptosis of epithelial cells. Epithelial cells were exposed to low oxygen for 3 h and subsequently stained with annexin V staining. Apoptotic cells were detected by fluorescence microscopy. *A*) Percentage of breast epithelial cells undergoing apoptosis after exposure to normoxic and hypoxic conditions. Student's *t* test. $*P < 0.05$. *B*) Representative micrographs of hypoxia-induced apoptotic cells as detected by fluorescence microscopy for MDA-BM-231, T47D, MCF7, and MCF10a cells. Arrows: apoptotic cells. Mean ± SD. Scale bar, 50 μm.

cell-breast cancer cell hybrids indicated that hybrids can undergo DNA ploidy reduction and morphologic switching from mesenchymal-like to breast carcinoma–like. In addition, analysis of coding single-nucleotide polymorphisms by RNA sequencing showed genetic contributions from both fusion partners in the primary tumor and metastases (41).

Spontaneous fusion was also reported with other cancer cell types. A study showed that mesenchymal stem cells

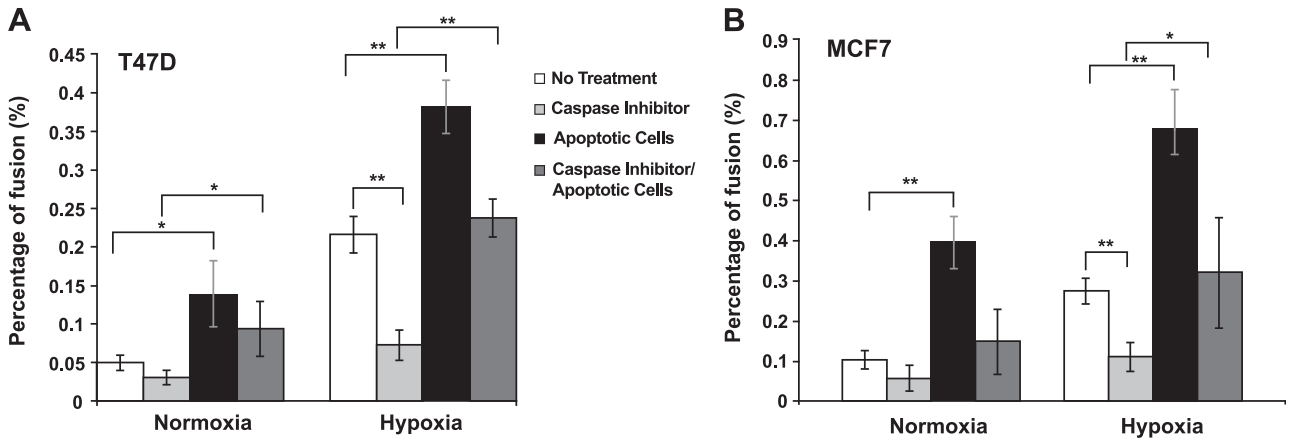

Figure 4. Apoptosis enhances fusion between MSCs and T47Ds or MSCs and MCF7s. *A*) Fusion between MSCs and T47Ds was impaired by treatment with the pan-caspase inhibitor Z-VAD-FMK. However, addition of apoptotic cells enhanced fusion between the 2 cell types and even rescued the fusion inhibited by the caspase inhibitor. *B*) Fusion between MSCs and MCF7s was impaired by the pan-caspase inhibitor z-VAD-FMK, as well. However, addition of apoptotic cells enhanced fusion between the 2 cell types and also rescued the fusion inhibited by the caspase inhibitor (mean ± SD). ANOVA with Tukey's HSD *post hoc* test. $*P < 0.05$; $**P < 0.01$.

could spontaneously fuse with lung cancer cells *in vitro*. Fusion was also detected *in vivo* when freshly mixed lung cancer cells and MSCs were xenografted by subcutaneous injection into nonobese diabetic (NOD)/SCID immunodeficient mice (42). The hybrids formed acquired increased motility and invasiveness and were found to contribute to highly malignant subpopulations with both epithelial-to-mesenchymal and stem cell–like properties (42). In addition, cells of a melanoma clone (wild-type for tyrosinase, *C/C*) implanted into BALB/c nu/nu mice (homozygous mutation for albino tyrosinase, *c/c*) developed massive pulmonary metastases a few weeks later. Analysis of chromosomes of cells from the metastatic tumors showed that most clones had acquired the *c* allele (same as that of the BALB/c recipient) while maintaining the *C* allele. Thus, lung metastases primarily comprised host-tumor hybrids and it is interesting that these hybrids expressed the same traits of enhanced motility and MSH/BMX responsiveness as *in vitro*–derived melanoma-macrophage hybrids (4).

Clinical studies have confirmed the presence of cell fusion in tumors as well, as found in patients who had undergone hematopoietic stem cell transplantation, with subsequent development of tumors showing evidence of donor genes in their cells (22, 43). In addition, a new report used STR polymorphism and forensic genetic techniques to suggest that a metastatic melanoma lesion in a patient arose from the fusion between a bone marrow–derived cell the patient received as a transplant and a tumor cell (44).

Our findings are in line with those in previous studies supporting the notion that cell fusion contributes to the ability of cancer cells to disseminate and perhaps metastasize. Moreover, a previous study showed that the motility-associated integrin subunits α2, α3, α5, α6, αv, β1, and β3, which are involved in the migration of leukocytes and cancer cells, were significantly upregulated in metastatic macrophage-melanoma hybrids compared with parental melanoma cells (45). A more recent study also indicated that fusion between cancer cells (ovarian and lung) and hematopoietic cells of the myeloid lineage gave rise to hybrids expressing significantly higher levels of the promigratory marker CXC chemokine receptor (CXCR)-4, which was conferred by the parental myeloid cells (46). Collectively, the increase in motility-associated integrin subunits and CXCR4 levels in hybrids may equip them with superior migratory potential and help their dissemination to various secondary organs, therefore explaining how fusion could provide a means by which adherent cancer cells acquire new qualities necessary to form metastases (*i.e.,* enhanced motility and matrix degradation) under conditions conducive to hematopoietic survival and later resume tumorlike activities (*i.e.,* rapid proliferation) under conditions conducive to epithelial survival. Cancer cell fusion may also provide a nonmutational mechanism that could contribute to aberrant gene expression patterns and give rise to highly malignant subpopulations with properties of cancer stem cells.

In contrast, a study showed that fusion between hematopoietic stem cells and human esophageal cancer cells slowed the growth speed of esophageal cancer cells and decreased the clone formation ability and tumorigenicity in NOD/SCID mice (47). So far, only the tumorigenicity of some hybrids that were formed *in vitro* between normal epithelial cells or fibroblasts and tumorigenic cancer cells has been shown to be generally suppressed compared to the parental cancer cells (48–50), probably because of activation of some tumor-suppressor genes, although some exceptions to this observation have been shown (20). The hybrids obtained in this way may deserve in depth analyses to understand the involvement of fusion in their tumor suppressor phenotype.

MSCs are known to be present in both healthy and cancerous breast tissue (51, 52) and have even been observed trafficking directly from bone marrow to sites of primary breast tumors (53). These studies have implicated MSCs in encouraging the migration and metastasis of primary tumors, primarily through MSC secretion of chemotactic cytokines such as CCL5. Our results suggest that cell fusion may be an additional mechanism by which MSCs encourage breast cancer metastasis.

Although cell fusion occurs naturally in many fundamental biologic processes [homotypic fusion of myoblasts,

placental trophoblasts (21), and macrophages (21, 54) and heterotypic gamete fusion (21)] and some pathologic conditions [viral-mediated fusion (55)], the mechanisms by which this phenomenon occurs are poorly understood, especially in the context of cancer. Studies have shown that cancer cell fusion can be induced by viruses (2, 56) or cell-cell invasion mechanisms of cellocytosis (cell-cell internalization) (54, 57) or entosis (a nonapoptosis cell death mechanism) (58). A recent work, however, identified apoptotic cells as a new type of cue that induces signaling *via* the PtdSer receptor BAI1 to promote fusion of healthy myoblasts (18). The 7-transmembrane protein BAI1 (a member of the adhesion type GPCR family) mediates recognition of PtdSer on apoptotic cells and signals through the ELMO/Dock180/Rac1 pathway to enhance mammalian myoblast fusion. In that study, blocking apoptosis potently impaired myoblast fusion, and adding back apoptotic myoblasts restored it (18). Our findings are consistent with those in that study, as addition of apoptotic cells significantly increased cell fusion between MSCs and T47D breast cancer cells. We also observed reduction of cell fusion when apoptosis was inhibited, but this reduction was rescued by supplementation of Z-VAD-FMK-treated cocultures with apoptotic cells. It has been shown that myoblasts and macrophages use some of the same molecular components in fusion, including the Dock180 protein (59), suggesting that PtdSer-BAI1 signaling is used by other cell types for fusion in addition to myoblasts. Moreover, BAI1 is well expressed in other cell types, including breast cancer cells and bone marrow–derived cells. These findings, together with our data, indicate that the mechanism by which cancer cells fuse with MSCs can involve apoptosis and may operate *via* PtdSer-BAI1 signaling.

We therefore propose that hypoxia-induced apoptosis in primary tumors stimulates cell fusion between MSCs and tumor cells, resulting in hybrids that exhibit both parental properties (mobility and proliferation) that enable their dissemination and new tumor growth at distant sites or metastases. *In vivo* studies addressing whether metastasis can in fact be initiated by fusion of cancer cells will complement this study. Investigating the involvement of BAI1 in cancer cell fusion will help delineate the molecular mechanisms of fusion of cancer cells and potentially the mechanisms of metastasis development. In the long run, this study may provide new strategies for developing alternate drugs for cancer treatment and preventing metastatic spread. FJ

The authors thank Dr. Peiman Hematti (Division of Hematology and Oncology, University of Wisconsin, Madison, WI, USA) for the gift of MSCs, Dr. Thomas Kerppola (Professor of Biological Chemistry, University of Michigan, Ann Arbor, MI, USA) for the gift of BiFC constructs, Louis Prahl (University of Minnesota, Minneapolis, MN, USA) for help with the time-lapse microscopy, Dr. Philip Jung (University of Minnesota, Minneapolis, MN, USA) for help with ANOVA with Tukey's HSD *post hoc* test, and Brian Freeman (University of Minnesota, Minneapolis, MN, USA) for help with the coculture analyses. This work was supported by U.S. Department of Defense, Idea Award 11-1-0205. F.N.K. participated in the study design, performed all the experiments, analyzed the data, and participated in writing the manuscript. T.H. developed molecular tools and protocols for coculture studies and helped to edit the manuscript. C.A. participated in study design, offered helpful consultation in the course of the studies, and helped to edit the manuscript. B.O. participated in study design, offered helpful consultation in the course of the studies and participated in writing the manuscript. All authors read and approved the final manuscript. The authors declare no conflicts of interest.

REFERENCES

1. Aichel, O. (1911) Über Zellverschmelzung mit qualitativ abnormer Chromosomenverteilung als Ursache der Geschwulstbildung *[*About *c*ell merging with high abnormal chromosome distribution as a cause of tumor formation*]*, Engelmann, Leipzig, Germany
2. Duelli, D. M., Padilla-Nash, H. M., Berman, D., Murphy, K. M., Ried, T., and Lazebnik, Y. (2007) A virus causes cancer by inducing massive chromosomal instability through cell fusion. *Curr. Biol.* **17**, 431–437
3. Pawelek, J. M., and Chakraborty, A. K. (2008) The cancer cell: leukocyte fusion theory of metastasis. *Adv. Cancer Res.* **101**, 397–444
4. Chakraborty, A. K., Sodi, S., Rachkovsky, M., Kolesnikova, N., Platt, J. T., Bolognia, J. L., and Pawelek, J. M. (2000) A spontaneous murine melanoma lung metastasis comprised of host x tumor hybrids. *Cancer Res.* **60**, 2512–2519
5. Oren-Suissa, M., and Podbilewicz, B. (2007) Cell fusion during development. *Trends Cell Biol.* **17**, 537–546
6. Miyado, K., Yamada, G., Yamada, S., Hasuwa, H., Nakamura, Y., Ryu, F., Suzuki, K., Kosai, K., Inoue, K., Ogura, A., Okabe, M., and Mekada, E. (2000) Requirement of CD9 on the egg plasma membrane for fertilization. *Science* **287**, 321–324
7. Kaji, K., Oda, S., Miyazaki, S., and Kudo, A. (2002) Infertility of CD9-deficient mouse eggs is reversed by mouse CD9, human CD9, or mouse CD81; polyadenylated mRNA injection developed for molecular analysis of sperm-egg fusion. *Dev. Biol.* **247**, 327–334
8. Jégou, A., Ziyyat, A., Barraud-Lange, V., Perez, E., Wolf, J. P., Pincet, F., and Gourier, C. (2011) CD9 tetraspanin generates fusion competent sites on the egg membrane for mammalian fertilization. *Proc. Natl. Acad. Sci. USA* **108**, 10946–10951
9. Hashizume, H., Baluk, P., Morikawa, S., McLean, J. W., Thurston, G., Roberge, S., Jain, R. K., and McDonald, D. M. (2000) Openings between defective endothelial cells explain tumor vessel leakiness. *Am. J. Pathol.* **156**, 1363–1380
10. McDonald, D. M., and Foss, A. J. (2000) Endothelial cells of tumor vessels: abnormal but not absent. *Cancer Metastasis Rev.* **19**, 109–120
11. Morikawa, S., Baluk, P., Kaidoh, T., Haskell, A., Jain, R. K., and McDonald, D. M. (2002) Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors. *Am. J. Pathol.* **160**, 985–1000
12. Jezková, J., Nováková, O., Kolár, F., Tvrzická, E., Neckár, J., and Novák, F. (2002) Chronic hypoxia alters fatty acid composition of phospholipids in right and left ventricular myocardium. *Mol. Cell. Biochem.* **232**, 49–56
13. Van Blitterswijk, W. J., Hilkmann, H., and Storme, G. A. (1987) Accumulation of an alkyl lysophospholipid in tumor cell membranes affects membrane fluidity and tumor cell invasion. *Lipids* **22**, 820–823
14. Mondal Roy, S., and Sarkar, M. (2011) Membrane fusion induced by small molecules and ions. *J. Lipids* **2011**, 528784
15. Hafez, I. M., Ansell, S., and Cullis, P. R. (2000) Tunable pH-sensitive liposomes composed of mixtures of cationic and anionic lipids. *Biophys. J.* **79**, 1438–1446
16. Paltsyn, A. A., Manukhina, E. B., Goryacheva, A. V., Downey, H. F., Dubrovin, I. P., Komissarova, S. V., and Kubatiev, A. A. (2014) Intermittent hypoxia stimulates formation of binuclear neurons in brain cortex: a role of cell fusion in neuroprotection? *Exp. Biol. Med. (Maywood)* **239**, 595–600
17. Lazova, R., Chakraborty, A., and Pawelek, J. M. (2011) Leukocyte-cancer cell fusion: initiator of the Warburg effect in malignancy? *Adv. Exp. Med. Biol.* **714**, 151–172
18. Hochreiter-Hufford, A. E., Lee, C. S., Kinchen, J. M., Sokolowski, J. D., Arandjelovic, S., Call, J. A., Klibanov, A. L., Yan, Z., Mandell, J. W., and Ravichandran, K. S. (2013) Phosphatidylserine receptor BAI1 and apoptotic cells as new promoters of myoblast fusion. *Nature* **497**, 263–267
19. Trivedi, P., and Hematti, P. (2007) Simultaneous generation of CD34 + primitive hematopoietic cells and CD73+ mesenchymal stem cells from human embryonic stem cells cocultured with murine OP9 stromal cells. *Exp. Hematol.* **35**, 146–154
20. Defendi, V., Ephrussi, B., Koprowski, H., and Yoshida, M. C. (1967) Properties of hybrids between polyoma-transformed and normal mouse cells. *Proc. Natl. Acad. Sci. USA* **57**, 299–305

21. Aguilar, P. S., Baylies, M. K., Fleissner, A., Helming, L., Inoue, N., Podbilewicz, B., Wang, H., and Wong, M. (2013) Genetic basis of cell-cell fusion mechanisms. *Trends Genet.* **29**, 427–437
22. Yilmaz, Y., Lazova, R., Qumsiyeh, M., Cooper, D., and Pawelek, J. (2005) Donor Y chromosome in renal carcinoma cells of a female BMT recipient: visualization of putative BMT-tumor hybrids by FISH. *Bone Marrow Transplant.* **35**, 1021–1024
23. Kerppola, T. K. (2006) Visualization of molecular interactions by fluorescence complementation. *Nat. Rev. Mol. Cell Biol.* **7**, 449–456
24. Alvarez-Dolado, M., Pardal, R., Garcia-Verdugo, J. M., Fike, J. R., Lee, H. O., Pfeffer, K., Lois, C., Morrison, S. J., and Alvarez-Buylla, A. (2003) Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. *Nature* **425**, 968–973
25. Croce, C. M., Shander, M., Martinis, J., Cicurel, L., D'Ancona, G. G., Dolby, T. W., and Koprowski, H. (1979) Chromosomal location of the genes for human immunoglobulin heavy chains. *Proc. Natl. Acad. Sci. USA* **76**, 3416–3419
26. Erikson, J., Martinis, J., and Croce, C. M. (1981) Assignment of the genes for human lambda immunoglobulin chains to chromosome 22. *Nature* **294**, 173–175
27. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J. Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., and Cardona, A. (2012) Fiji: an open-source platform for biological-image analysis. *Nat. Methods* **9**, 676–682
28. Kemp, K., Wilkins, A., and Scolding, N. (2014) Cell fusion in the brain: two cells forward, one cell back. *Acta Neuropathol.* **128**, 629–638
29. Vaupel, P., and Harrison, L. (2004) Tumor hypoxia: causative factors, compensatory mechanisms, and cellular response. *Oncologist* **9**(Suppl 5), 4–9
30. Greijer, A. E., and van der Wall, E. (2004) The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis. *J. Clin. Pathol.* **57**, 1009–1014
31. Mekler, L. B. (1971) [Hybridization of transformed cells with lymphocytes as 1 of the probable causes of the progression leading to the development of metastatic malignant cells]. *Vestn. Akad. Med. Nauk SSSR* **26**, 80–89
32. Goldenberg, D. M. (1968) [On the progression of malignity: a hypothesis]. *Klin. Wochenschr.* **46**, 898–899
33. Berndt, B., Zänker, K. S., and Dittmar, T. (2013) Cell fusion is a potent inducer of aneuploidy and drug resistance in tumor cell/normal cell hybrids. *Crit. Rev. Oncog.* **18**, 97–113
34. Berndt, B., Haverkampf, S., Reith, G., Keil, S., Niggemann, B., Zänker, K. S., and Dittmar, T. (2013) Fusion of CCL21 non-migratory active breast epithelial and breast cancer cells give rise to CCL21 migratory active tumor hybrid cell lines. *PLoS ONE* **8**, e63711
35. Dittmar, T., Schwitalla, S., Seidel, J., Haverkampf, S., Reith, G., Meyer-Staeckling, S., Brandt, B. H., Niggemann, B., and Zänker, K. S. (2011) Characterization of hybrid cells derived from spontaneous fusion events between breast epithelial cells exhibiting stem-like characteristics and breast cancer cells. *Clin. Exp. Metastasis* **28**, 75–90
36. Ozel, C., Seidel, J., Meyer-Staeckling, S., Brandt, B. H., Niggemann, B., Zänker, K. S., and Dittmar, T. (2012) Hybrid cells derived from breast epithelial cell/breast cancer cell fusion events show a differential RAF-AKT crosstalk. *Cell Commun. Signal.* **10**, 10
37. Lu, X., and Kang, Y. (2009) Efficient acquisition of dual metastasis organotropism to bone and lung through stable spontaneous fusion between MDA-MB-231 variants. *Proc. Natl. Acad. Sci. USA* **106**, 9385–9390
38. Yang, J. J., Milton, M. N., Yu, S., Liao, M., Liu, N., Wu, J. T., Gan, L., Balani, S. K., Lee, F. W., Prakash, S., and Xia, C. Q. (2010) P-glycoprotein and breast cancer resistance protein affect disposition of tandutinib, a tyrosine kinase inhibitor. *Drug Metab. Lett.* **4**, 201–212
39. Yang, J. Y., Ha, S. A., Yang, Y. S., and Kim, J. W. (2010) p-Glycoprotein ABCB5 and YB-1 expression plays a role in increased heterogeneity of breast cancer cells: correlations with cell fusion and doxorubicin resistance. *BMC Cancer* **10**, 388
40. Mortensen, K., Lichtenberg, J., Thomsen, P. D., and Larsson, L. I. (2004) Spontaneous fusion between cancer cells and endothelial cells. *Cell. Mol. Life Sci.* **61**, 2125–2131
41. Rappa, G., Mercapide, J., and Lorico, A. (2012) Spontaneous formation of tumorigenic hybrids between breast cancer and multipotent stromal cells is a source of tumor heterogeneity. *Am. J. Pathol.* **180**, 2504–2515
42. Xu, M. H., Gao, X., Luo, D., Zhou, X. D., Xiong, W., and Liu, G. X. (2014) EMT and acquisition of stem cell-like properties are involved in spontaneous formation of tumorigenic hybrids between lung cancer and bone marrow-derived mesenchymal stem cells. *PLoS ONE* **9**, e87893
43. Chakraborty, A., Lazova, R., Davies, S., Bäckvall, H., Ponten, F., Brash, D., and Pawelek, J. (2004) Donor DNA in a renal cell carcinoma metastasis from a bone marrow transplant recipient. *Bone Marrow Transplant.* **34**, 183–186
44. Lazova, R., Laberge, G. S., Duvall, E., Spoelstra, N., Klump, V., Sznol, M., Cooper, D., Spritz, R. A., Chang, J. T., and Pawelek, J. M. (2013) A melanoma brain metastasis with a donor-patient hybrid genome following bone marrow transplantation: first evidence for fusion in human cancer. *PLoS ONE* **8**, e66731
45. Chakraborty, A. K., Pawelek, J., Ikeda, Y., Miyoshi, E., Kolesnikova, N., Funasaka, Y., Ichihashi, M., and Taniguchi, N. (2001) Fusion hybrids with macrophage and melanoma cells up-regulate N-acetylglucosaminyltransferase V, beta1-6 branching, and metastasis. *Cell Growth Differ.* **12**, 623–630
46. Ramakrishnan, M., Mathur, S. R., and Mukhopadhyay, A. (2013) Fusion-derived epithelial cancer cells express hematopoietic markers and contribute to stem cell and migratory phenotype in ovarian carcinoma. *Cancer Res.* **73**, 5360–5370
47. Fan, H., and Lu, S. (2014) Fusion of human bone hemopoietic stem cell with esophageal carcinoma cells didn't generate esophageal cancer stem cell. *Neoplasma* **61**, 540–545
48. Stanbridge, E. J. (1976) Suppression of malignancy in human cells. *Nature* **260**, 17–20
49. Harris, H. (1988) The analysis of malignancy by cell fusion: the position in 1988. *Cancer Res.* **48**, 3302–3306
50. Weinberg, R. A. (1991) Tumor suppressor genes. *Science* **254**, 1138–1146
51. Zhao, M., Sachs, P. C., Wang, X., Dumur, C. I., Idowu, M. O., Robila, V., Francis, M. P., Ware, J., Beckman, M., Rizki, A., Holt, S. E., and Elmore, L. W. (2012) Mesenchymal stem cells in mammary adipose tissue stimulate progression of breast cancer resembling the basal-type. *Cancer Biol. Ther.* **13**, 782–792
52. Karnoub, A. E., Dash, A. B., Vo, A. P., Sullivan, A., Brooks, M. W., Bell, G. W., Richardson, A. L., Polyak, K., Tubo, R., and Weinberg, R. A. (2007) Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature* **449**, 557–563
53. Liu, S., Ginestier, C., Ou, S. J., Clouthier, S. G., Patel, S. H., Monville, F., Korkaya, H., Heath, A., Dutcher, J., Kleer, C. G., Jung, Y., Dontu, G., Taichman, R., and Wicha, M. S. (2011) Breast cancer stem cells are regulated by mesenchymal stem cells through cytokine networks. *Cancer Res.* **71**, 614–624
54. Vignery, A. (2005) Macrophage fusion: are somatic and cancer cells possible partners? *Trends Cell Biol.* **15**, 188–193
55. Podbilewicz, B. (2014) Virus and cell fusion mechanisms. *Annu. Rev. Cell Dev. Biol.* **30**, 111–139
56. Pawelek, J. M., and Chakraborty, A. K. (2008) Fusion of tumour cells with bone marrow-derived cells: a unifying explanation for metastasis. *Nat. Rev. Cancer* **8**, 377–386
57. Vignery, A. (2005) Macrophage fusion: the making of osteoclasts and giant cells. *J. Exp. Med.* **202**, 337–340
58. Overholtzer, M., Mailleux, A. A., Mouneimne, G., Normand, G., Schnitt, S. J., King, R. W., Cibas, E. S., and Brugge, J. S. (2007) A nonapoptotic cell death process, entosis, that occurs by cell-in-cell invasion. *Cell* **131**, 966–979
59. Pajcini, K. V., Pomerantz, J. H., Alkan, O., Doyonnas, R., and Blau, H. M. (2008) Myoblasts and macrophages share molecular components that contribute to cell-cell fusion. *J. Cell Biol.* **180**, 1005–1019

Received for publication February 9, 2015.
Accepted for publication June 2, 2015.